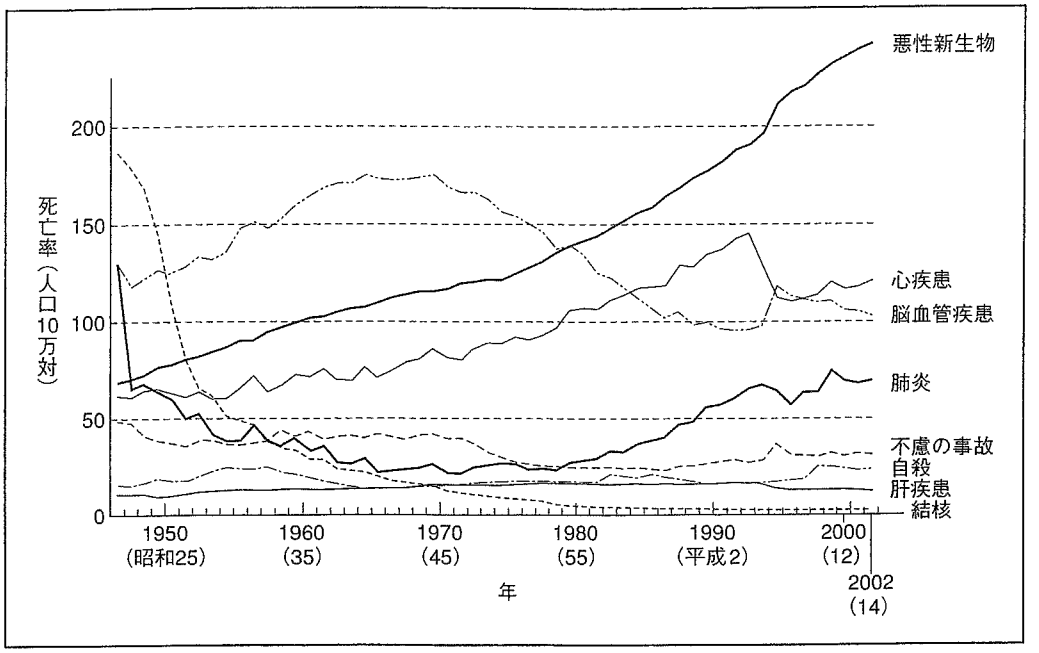

図2．主要死因別にみた死亡率の年次推移

資料：厚生労働省大臣官房統計情報部「人口動態統計」
注：死因分類等の改正により，死因の内容に完全な一致をみることはできない。
平成13年までは確定数，平成14年は概数である。

っている。また，2001年の国民生活基礎調査によると，わが国の要介護者の割合を疾患別にみると，脳血管疾患が全体の27.7%と最も高く，次いで高齢による衰弱，骨折・転倒，認知症の順である（**図3**）[7][8]。高齢社会を迎えた現在，脳血管障害や骨関節疾患，認知症に起因する障害を有する高齢者の数は増加しており，リハビリを必要とする高齢者は，ますます増加する可能性が高いと考えられる。

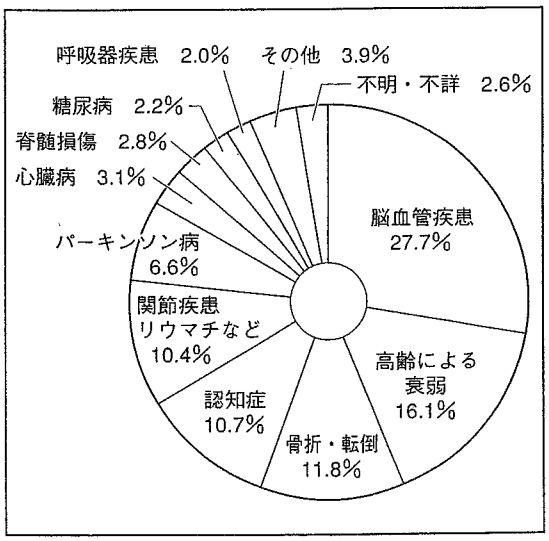

図3．介護が必要となった主な原因
統計情報部「平成13年国民生活基礎調査」

Ⅱ．高齢者リハビリテーションの理念

高齢者リハビリでは，対応する疾患や方法論も当然変化している。単に高齢発症による疾患の障害に対応するだけでなく，高齢者特有の特性を理解してリハビリを行う必要がある。高齢者リハビリの中では，従来のリハビリの定義である“障害者を可能な限り身体的，精神的，社会的，職業的および経済的に最高度の有用性を獲得するように回復させることである”をこのまま高齢者にあてはめることは困難である。高齢者では，社会的，職業的または経済的に有用性をもたせるためのリ

ハビリは，ごく限られたものが対象で，大多数の高齢者では身体的および精神的な機能の回復を最大限に図り，可能な限り独立して生活し得る能力を取り戻すことが目標となる。このために高齢者リハビリの流れから，第1に寝たきりや要介護状態を予防する予防的リハビリ，第2に疾病の治療とともに早期に開始される急性期リハビリ，第3に急性期から機能回復を目指した回復期リハビリへのスムースな移行，第4に地域との連携が重要で，維持期リハビリが必要となる。以下に高齢者リハビリの理念について概説する。

1．高齢者の残存機能を引き出し，自立した生活を目標とする

従来の医療は，臓器疾患の治療を第一義的に考えた延命のための医療が中心で，急性期疾患の治療や救命に多大な貢献を果たしてきたが，高齢者医療ではこの先進的臓器別診療では高齢患者のQOLを損なう場合すらある。例えば，高齢者医療では複数の疾患を有していたり，慢性疾患を合併していた場合，臓器別の診療により単一の疾患の治療に専念するばかりに，関節拘縮，筋力低下などの廃用症候群を引き起こし，かえって生活機能低下をもたらし，疾患は治ったが自宅での自立した生活ができなくなるという高齢者にとって不幸な事態を生じることがある。そのためには高齢者のQOLの向上を目指し，自立した生活を送ることに着目する医療の必要性が問われるようになってきた。高齢者医療では，単なる臓器の疾患の診断と治療ではなく全人的な包括医療が必須であり，患者の日常生活動作，精神機能，および社会的状況の三つを総合的に評価して効果的な医療を行うことが必要である。従来よりリハビリは，包括医療を実践してきた診療科であり，医療の中に日常生活動作の観点を取り入れてきた学問である。そのためリハビリでは，高齢者の残存機能を引き出し，生活の自立機能の障害予防を最終目的とした医療は得意とする領域であり，高齢者医療の中で自立機能障害の予防を果たすのにリハビリの位置づけは非常に重いといえる。

2．予防医学としてのリハビリテーションを重視する

加齢とともに，さまざまな疾患や障害を有するようになるが，高齢者が障害に悩まされることなく，老後に活力のある生活を送れるようにするためには，高齢期，特に75歳以上の後期高齢者に発症する疾患や障害の発症をいかに予防していくかが特に重要な課題となる。成人期からの不適切な生活習慣が主要な発症原因であるといわれている脳血管障害，虚血性心疾患，閉塞性動脈硬化症などの動脈硬化性血管障害や骨粗鬆症などは，不適切な生活習慣を適正化することにより，これらの疾患の多くを予防あるいは発症を遅らせることができる[2]。また現在，介護を必要とするようになる原因としては，脳血管疾患，高齢による衰弱，転倒・骨折，認知症が頻度の高いものであり，これらを予防することが，要介護状態を予防することになる。そのために，高齢による衰弱を除いた脳血管疾患，転倒による骨折，認知症などの予防対策が必要である。要介護状態および寝たきりを予防するためにも，リハビリの果たす役割は重要である。しかし，リハビリの手法も従来の方法では対応困難な疾患もみられる。例えば，認知症のリハビリはまだ十分に確立しているとは言い難い。

3．高齢者の特性を踏まえたリハビリテーションが必要性である[3]

リハビリを行っていく場合，高齢者の特性を理解したうえで実施していかなくてはいけない。高齢者に多発する疾患の多くは老化を基盤にしており，いくつかの特徴を兼ね備えている。高齢者では多臓器にわたる疾患が認められ，症状が非定型的である。そして慢性化しやすいし，機能障害につながりやすく，合併症を併発しやすい。さらに社会的要因や環境により症状が変動しやすい。例えば，高齢者が入院により夜間せん妄を引き起こすことがある。リハビリを実施するうえで注意を払う必要があることは，まず第1に疾病の治療とともに身体活動性を保つことを優先することであ

る。高齢者ではいったん疾病に罹患すると若年者に比べ重症化，長期化しやすく，廃用症候群に陥りやすい。そのために，疾病の治療だけが優先されるのではなく，日常生活動作能力を維持し，改善しながらQOLを高めることが求められる。高齢者医療では病気は治ったが入院前の生活に戻れないようではいけないのである。また高齢者には，一度に複数の慢性疾患を抱えている可能性がある。医療技術の進歩により，数々の病気を早期に発見，治療できるようになったが，すべての疾患を完全治癒にもっていくことが困難な場合が多い。疾病を根治するのではなく，慢性疾患をもちながら，自立した生活を維持することも大切である。高齢者リハビリでは，複数存在する慢性疾患の増悪や合併症の発症に注意を払いながらリハビリを実施していく必要がある。

4．高齢者医療では，より一層のチーム医療が必要とされている

高齢者医療は，包括的医療といわれている。高齢者はいったん疾病に罹患すると，廃用症候群に陥りやすく，疾病の治療ばかりに目を向けていると，すぐに日常生活動作能力が低下し，入院も長期化する可能性が高い。そのため，高齢者リハビリでは，より一層早期からのリハビリを開始することが必要で，生活機能を維持するために，医師のみならず，看護師，理学療法士，作業療法士，言語聴覚士，薬剤師，ケースワーカーと協力しあい，情報交換を密に行いながら，疾病の治療とともに日常生活動作能力を保持しなければならない。チーム医療を進める方法として，さまざまな専門家やコメディカルが一同に会して合同カンファレンスを開くのが一つの方法である。いくら熟練した医師でも，すべての専門性を網羅することは不可能であり，時間と能力には限りがあるので，周囲に専門職が複数いて，互いのコミュニケーションをよくすることで，よりよい医療が患者に提供できる。各科医師とコメディカルが情報を共有し，適切に育成された人的資源を有効に活用する。

表1．老年症候群

意識障害
認知症，せん妄
不眠，うつ状態
めまい
言語障害，聴覚障害，視力障害
骨関節変形，骨粗鬆症，骨折
尿失禁，夜間頻尿
誤嚥，便秘，下痢
脱水，発熱
低体温
浮腫，肥満，るいそう，低栄養
褥瘡
喘鳴，喀痰咳嗽，呼吸困難
手足のしびれ，間欠性跛行
動脈硬化，不整脈
痛み
出血傾向，吐血，下血
ADL低下

5．高齢者リハビリテーションでは，特有の疾患を対象とする[10)11)]

老化により，骨関節・筋などの運動器，呼吸・循環器系，神経系，精神機能，代謝機能など多くの臓器に形態的変化と機能的変化をもたらす。これらのことが，高齢者に特有の疾病を発生させることになる。具体的には，高齢者では脳血管障害，認知症，パーキンソン症候群を中心とした中枢神経障害，転倒による大腿骨頸部骨折，脊椎圧迫骨折，変形性関節症，変形性脊椎症，骨粗鬆症などの整形外科疾患，肺炎や慢性呼吸不全などの呼吸器疾患，心筋梗塞や胸部手術後，血管手術，悪性腫瘍などの腹部手術後，糖尿病による神経障害もリハビリ対象疾患として多くみられる。また高齢者に多くみられ，原因はさまざまであるが治療と同時に介護およびケアを必要とする一連の症状や所見を老年症候群と呼称する。具体的な老年症候を表1に示す[12)]。これらの老年症候を多くもつことはリハビリの阻害因子を多く抱えていることになり，リハビリを施行するうえで注意を払わなければならないものである。その中でも嚥下障害，骨折などは，リハビリ対象疾患の大きな部分を占

めている。

Ⅲ．高齢者リハビリテーションの課題

1．予防的リハビリテーションへの取り組み

寝たきりまたは要介護状態の予防に対しても，積極的に対応していかなければならない。例えば，認知症の進行を予防する有効なリハビリプログラムを確立することも急務である。認知症高齢者に対する非薬物的療法としては，具体的には，回想法，リアリティオリエンテーション，行動療法，sensory stimulation，音楽療法，理学療法(筋力強化，バランス訓練，関節可動域訓練)，作業療法（家事・家庭内役割作業，手工芸・工作)，レクリエーション，園芸療法，演芸療法，社会心理療法，散歩，ラジオ体操，リズム体操，民謡体操，ストレッチ体操，肩こり体操，ダンスなどがあり，環境の整備，介護者への教育・指導なども含めると多岐にわたる。しかしながら，まだ有効性が確立した認知症に対するリハビリプログラムはみられない[13]。また，大腿骨頸部骨折の90%は転倒に起因するともいわれるが，高齢者の転倒には筋力の低下，視力の低下，認知症，生活様式など多くの要因が関与している。大腿骨頸部骨折患者の生命予後はよいが，約半数は歩行能力が低下し，約20%は寝たきり状態に陥るといわれている。転倒予防が骨折予防につながると考えられ，この目的で転倒予防および骨粗鬆症に対してもリハビリは対応していかなければいけない[14][15]。高齢者リハビリでは，より一層の予防目的のリハビリが重視されることになる。

2．高齢者の特性を踏まえたリハビリテーションプログラムの確立[16]

高齢者は，体力低下を伴っていることが多く，耐久性も低下しているために，長時間でかつ複雑な訓練内容は導入できないことがあり，単純で慣れた動作を中心とした訓練計画を立てる必要がある。例えば高齢者では，加齢とともに減少する筋肉量や平衡感覚機能は容易に回復しない。また，筋力増強の重要性は認識されていても，高齢者が無理なく行える筋力増強の方法はまだ確立されていない。ただ高齢者では，運動量の多い激しい筋力トレーニングが必要なわけではない。各自が自分に合った運動をして，身体活動性を維持すればよい。高齢者には高齢者に適したリハビリプログラムが必要である。また高齢者では，他の年齢層に比べライフスタイルの違い，住む環境状況の違い，過去に経験してきたことの違いにより個人差が非常に大きいことと，家族制度など社会構造の変化がきて，家族構成上核家族化が進み，単独世帯，夫婦のみの世帯，夫婦ともに65歳以上の世帯などが増加していて，家庭での介護能力が減少している現状がある。それゆえに高齢者のリハビリを実施するうえで，社会的背景が個人で大きく異なるため部分的で一方的な見方では偏りがあり，広い視野に立って，リハビリプログラムの立案と最終的な目標を個別に設定する必要がある。

3．地域連携システムの確立

高齢者医療では，病院と地域の連携が密接である必要がある。このためには，医療と看護，介護の垣根を越えた連携が必要である。日本ではリハビリも多様化しており，医療保険下で行われる入院および通院でのリハビリおよび訪問リハビリと訪問看護があり，介護保険の導入後，特に訪問リハビリ，通所リハビリなど，医療で行われるリハビリとの境界が不明瞭となっている。高齢者の介護状態の軽減や在宅での自立した日常生活を重視するためには，高齢者が長年住み慣れた地域において満足できるリハビリが受けられる体制をつくる必要があり，医療保険と介護保険でのリハビリの役割分担を明確にして，スムースな移行ができるように体制を整える必要がある[17]。

4．証拠に基づいたリハビリテーション

医学界では，証拠に基づいた医療が求められており，リハビリ医学でも例外でなく，適切な無駄のない医療は，客観的な証拠があって初めて可能となる。客観的な証拠とは，リハビリとしてどの種類の運動を，どの頻度で，どのくらいの期間行

うと最良の効果が得られるかを科学的に明らかにすることである。例えば，高齢者での効果的な筋力強化のプログラムや，認知症に対する効果的なプログラムに対する証拠はまだ不十分であり，今後の報告が待たれるところである。

5．最新の医療に対応したリハビリテーションの必要性

現代医療は着実に進歩しており，高齢者にもその適応範囲は徐々に広がっていくことが予想される。先端医療としては，皮膚，筋肉，骨，軟骨などの移植医療，ES細胞や組織幹細胞を用いた再生医療，遺伝子治療など急速な医療の進歩がみられていく中で，それに応じたリハビリプログラムなどの開発が必要と考えられる。

おわりに

高齢化社会を迎えて，老化によりさまざまな疾患や障害を有する可能性が高くなり，疾患を治療するためにリハビリを含めた医療的ニーズはさらに高まることが推測できる。その一方で，医療費の高騰という社会・経済的な問題が起きているのも事実であり，医療費を抑えるためにも医療供給側は，より的確な治療を選択する必要があると同時に，疾患の治療だけでなく生活自立支援，健康増進や予防医学の側面にも積極的に介入する必要がある。リハビリの立場としては，これら生活自立支援，健康増進や予防医学にも対応していかなければならないために，求められる役割は非常に重要である。

文　献

1) 折茂　肇：21世紀の老人医療のあり方とその将来展望．Geriatric Medicine 37(3)：333－337, 1999.
2) 折茂　肇：序論．老年医療の歩みと展望，日本老年医学会編，メジカルビュー社，2003，pp 2－5.
3) 黒川幸雄，大西秀明：高齢社会と理学療法の展望．高齢者の理学療法，森本　榮，黒川幸雄編，三輪書店，2002，pp 2－6.
4) 井口昭久：老年医学とは何か．日老医誌 40：439－444，2003.
5) 内閣府（編）：平成15年版高齢社会白書．2004，p13.
6) 厚生労働省（監修）：厚生労働白書．平成15年版，活力ある高齢者像と世代間の新たな関係の構築，2004，p366.
7) 生活情報センター（編）：介護サービス統計資料年報2003，文栄社，2003，p45.
8) 柳澤信夫：高齢化社会における寝たきり予防の重要性．寝たきりの予防と治療，財団法人長寿科学振興財団編，保健同人社，2001，pp 9－16.
9) 下方浩史：高齢者の疫学．これからの老年学，井口昭久編，名古屋大学出版会，2000，pp41－56.
10) 鳥羽研二：高齢者の特徴と診療上の留意事項．老年医学，萩原俊男編，朝倉書店，2003，pp24－34.
11) 村上恵一：身体疾患のリハビリテーション．老年者のリハビリテーション，平井俊策，江藤文夫編，ワールドプランニング，1997，pp 9－15.
12) 鳥羽研二：老年症候群．老年医学テキスト，日本老年医学会編，メジカルビュー社，2002，pp98－103.
13) 長屋政博：痴呆疾患における理学療法の意義．老年期痴呆の克服をめざして，財団法人長寿科学振興財団編，保健同人社，2002，pp251－257.
14) 長屋政博，原田　敦，奥泉宏康：高齢者の骨折と予防のための運動療法．現代医学 45：329－333，1997.
15) 長屋政博，荒川幸子：転倒予防教室の効果．愛知県理学療法士会誌 15（1）：1－8，2003.
16) 長屋政博：リハビリテーション．Geriatric Medicine 39（11）：1757－1764，2001.
17) 石川　誠：リハビリテーション．寝たきりの予防と治療，財団法人長寿科学振興財団編，保健同人社，2001，pp117－132.

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Molecular and Cellular Endocrinology 220 (2004) 89–95

MCE
Molecular and Cellular Endocrinology

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Involvement of SAPK/JNK in prostaglandin E_1-induced VEGF synthesis in osteoblast-like cells

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Received 21 October 2003; accepted 25 March 2004

Abstract

We previously reported that prostaglandin E_1 (PGE_1) activates both p44/p42 mitogen-activated protein (MAP) kinase and p38 MAP kinase via cAMP-dependent protein kinase in osteoblast-like MC3T3-E1 cells, and that p38 MAP kinase but not p42/p44 MAP kinase is involved in PGE_1-induced synthesis of vascular endothelial growth factor (VEGF). In the present study, we investigated the involvement of stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) in the PGE_1-induced VEGF synthesis in MC3T3-E1 cells. PGE_1 induced the phosphorylation of SAPK/JNK. SP600125, a specific inhibitor of SAPK/JNK, markedly reduced the PGE_1-induced VEGF synthesis. Forskolin, a direct activator of adenylyl cyclase, elicited the phosphorylation of SAPK/JNK, and 8bromo-cAMP, a plasma membrane-permeable cAMP analogue-stimulated VEGF synthesis was significantly reduced by SP600125. SP600125 suppressed the PGE_1-induced phosphorylation of SAPK/JNK without affecting the phosphorylation of p38 MAP kinase induced by PGE_1. The phosphorylation of c-Jun induced by PGE_1 was also inhibited by SP600125. SB203580, a p38 MAP kinase inhibitor, failed to reduce the PGE_1 induced phosphorylation of SAPK/JNK. A combination of SP600125 and SB203580 suppressed the PGE_1-stimulated VEGF synthesis in an additive manner. These results strongly suggest that PGE_1 activates SAPK/JNK in osteoblasts, and that SAPK/JNK plays a part in PGE_1-induced VEGF synthesis.

Keywords: PGE_1; VEGF; SAPK/JNK; Osteoblast

1. Introduction

Vascular endothelial growth factor (VEGF) has been characterized as a heparin-binding angiogenic growth factor displaying high specificity for vascular endothelial cells (Ferrara and Davis-Smyth, 1997). It is well recognized that VEGF, which is produced and secreted from a variety of cell types, increases capillary permeability and stimulates proliferation of endothelial cells (Ferrara and Davis-Smyth, 1997). As for bone metabolism, it has been shown that inactivation of VEGF causes complete suppression of blood vessel invasion concomitant with impaired trabecular bone formation and expansion of hypertrophic chondrocyte zone in mouse tibial epiphyseal growth plate (Gerber et al., 1999). Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, the former responsible for bone formation and the latter for bone resorption (Nijweide et al., 1986). Accumulating evidence indicates that osteoblasts among bone cells produce and secrete VEGF in response to various humoral factors (Goad et al., 1996; Wang et al., 1996; Ferrara and Davis-Smyth, 1997; Schalaeppi et al., 1997). During bone remodeling, capillary endothelial cells provide the microvasculature, and osteoblasts and osteoprogenitor cells, which locally proliferate and differentiate into osteoblasts, migrate into the resorption lacuna. It is currently recognized that the activities of osteoblasts, osteoclasts, and capillary endothelial cells are closely coordinated via humoral factors as well as by direct cell-to-cell contact, and these cells cooperatively regulate bone metabolism (Erlebacher et al., 1995). Based on these findings, there

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doi:10.1016/j.mce.2004.03.010

is no doubt that VEGF secreted from osteoblasts plays a pivotal role in the regulation of bone metabolism. However, the exact mechanism behind VEGF synthesis in osteoblasts has not yet been fully clarified.

Prostaglandins (PGs) are well known as autocrine/paracrine modulators of osteoblasts, and play important roles in their cell functions (Nijweide et al., 1986; Smith, 1986). Among them, PGE_1 reportedly stimulates cyclic AMP (cAMP) production and induces alkaline phosphatase activity, a marker of osteoblast phenotype (Robinson et al., 1973), in osteoblasts (Pilbeam et al., 1996). It has been shown that PGE_1 increases the levels of mRNA for VEGF and produces VEGF in primary cultured rat calvaria cells and RCT-3 osteoblast-like cells, and that cAMP mediates the synthesis of VEGF (Harada et al., 1994). The mitogen-activated protein (MAP) kinase superfamily is well-recognized to play crucial roles in the intracellular signaling of variety of agonists (Widmann et al., 1999). Three MAP kinases, p44/p42 MAP kinase, p38 MAP kinase, and stress-activated protein kinase/c-Jun N-terminal kinase (SAPK/JNK) are known as central elements used by mammalian cells to transduce the various messages (Widmann et al., 1999). We previously reported that PGE_1 activates both p44/p42 MAP kinase and p38 MAP kinase via cAMP-dependent protein kinase (protein kinase A) in osteoblast-like MC3T3-E1 cells, and that p38 MAP kinase but not p44/p42 MAP kinase takes part in the VEGF synthesis induced by PGE_1 (Tokuda et al., 2001). However, the exact roles of SAPK/JNK in osteoblasts have not yet been clarified.

In the present study, we investigated whether SAPK/JNK is involved in the PGE_1-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. We here show that PGE_1 activates SAPK/JNK as well as p44/p42 MAP kinase and p38 MAP kinase in these cells, and that SAPK/JNK plays a part in the PGE_1 induced VEGF synthesis in addition to p38 MAP kinase.

2. Materials and methods

2.1. Materials

PGE_1, forskolin and 8bromo-cAMP were obtained from Sigma (St. Louis, MO). Mouse VEGF enzyme immunoassay kit was purchased from R & D Systems Inc. (Minneapolis, MN). SP600125 and SB203580 were obtained from Calbiochem-Novabiochem Co. (La Jolla, CA). Phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p38 MAP kinase antibodies, p38 MAP kinase antibodies, phospho-specific c-Jun antibodies and c-Jun antibodies were purchased from New England BioLabs Inc. (Beverly, MA). ECL Western blotting detection system was purchased from Amersham Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. PGE_1 and forskolin were dissolved in ethanol. SP600125 or SB203580 were dissolved in dimethyl sulfoxide (DMSO). The maximum concentration of ethanol or DMSO was 0.1%, which did not affect the assay for VEGF or the analysis of MAP kinases.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells derived from newborn mouse calvaria (Sudo et al., 1983) were maintained as previously described (Kozawa et al., 1992). Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37 °C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm diameter dishes or 90-mm diameter dishes in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h.

2.3. VEGF assay

The cultured cells were stimulated by PGE_1 or 8bromo-cAMP in 1 ml of α-MEM containing 0.3% FCS for the indicated periods. When indicated, the cells were pretreated with SP600125 or SB203580 for 60 min. The reaction was terminated by collecting the medium, and VEGF in the medium was measured by a VEGF enzyme immunoassay kit.

2.4. Analyses of MAP kinases and c-Jun

The cultured cells were stimulated by PGE_1 or forskolin in α-MEM containing 0.3% FCS for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lyzed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 × g for 10 min at 4 °C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by Laemmli (1970) in 10% polyacrylamide gel. Western blotting analysis was performed as described previously (Kato et al., 1996) by using phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific c-Jun antibodies, c-Jun antibodies, phospho-specific p38 MAP kinase antibodies or p38 MAP kinase antibodies with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by means of the ECL Western blotting detection system. When indicated, the cells were pretreated with SP600125 or SB203580 for 60 min.

2.5. Determinations

The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments Inc., Winooski, VT). The

densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

2.6. Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparisons between pairs, and a $P < 0.05$ was considered significant. All data are presented as the mean ± S.E.M. of triplicate determinations. Each experiment was repeated three times with similar results.

3. Results

3.1. Effect of PGE_1 on the phosphorylation of SAPK/JNK in MC3T3-E1 cells

We previously reported that PGE_1 activates p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells, and that p38 MAP kinase but not p44/p42 MAP kinase is involved in the VEGF synthesis induced by PGE_1 (Tokuda et al., 2001). To clarify whether PGE_1 activates SAPK/JNK in MC3T3-E1 cells, we examined the effect of PGE_1 on the SAPK/JNK phosphorylation. PGE_1 markedly induced the phosphorylation of SAPK/JNK in a time-dependent manner up to 60 min (Fig. 1).

3.2. Effect of SP600125 on the PGE_1-induced VEGF synthesis in MC3T3-E1 cells

To investigate whether SAPK/JNK is involved in the PGE_1-induced VEGF synthesis in MC3T3-E1 cells, we examined the effect of SP600125, a highly specific inhibitor of JNK on the synthesis (Bennett et al., 2001). SP600125, which alone hardly affected the basal level of VEGF, significantly reduced the PGE_1-induced VEGF synthesis (Fig. 2). The inhibitory effect of SP600125 on the PGE_1-induced VEGF synthesis was dose-dependent in the range between 1 and 50 μM (Fig. 3). The maximum inhibitory effect of SP600125 was observed at 50 μM, which caused about 85% reduction in the PGE_1-effect.

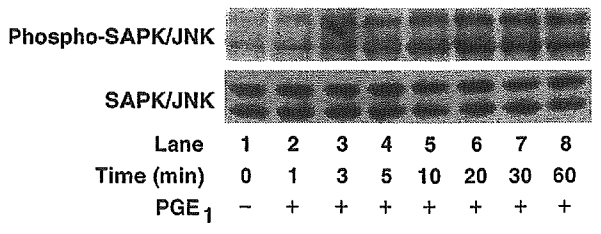

Fig. 1. Effect of PGE_1 on the phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were stimulated by 10 μM PGE_1 for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The upper bands and the lower ones indicated p54 and p46 SAPK/JNK, respectively. Lane 1, control cells.

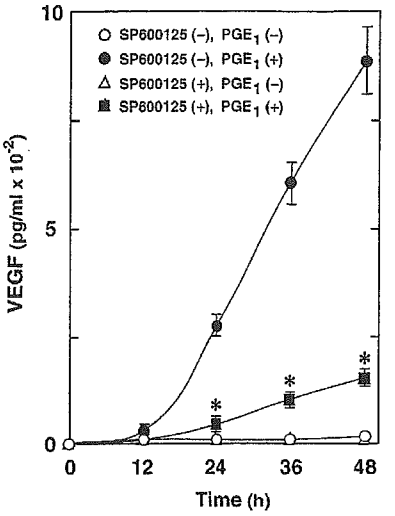

Fig. 2. Effect of SP600125 on the PGE_1-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM SP600125 (triangles) or vehicle (circles) for 60 min, and then stimulated by 10 μM PGE_1 (closed symbols) or vehicle (open symbols) for the indicated periods. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared to the value of PGE_1 alone.

3.3. Effects of SP600125 on the phosphorylation of SAPK/JNK or c-Jun induced by PGE_1 in MC3T3-E1 cells

We found that SP600125 truly inhibited the phosphorylation of SAPK/JNK induced by PGE_1 (Fig. 4). According to the densitometric analysis, SP600125 caused about 50% reduction of the PGE_1-effect on the SAPK/JNK phosphorylation.

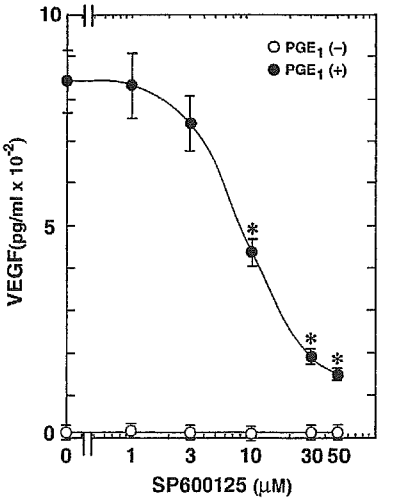

Fig. 3. Dose-dependent effect of SP600125 on the PGE_1-induced VEGF synthesis in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SP600125 for 60 min, and then stimulated by 10 μM PGE_1 (closed circles) or vehicle (open circles) for 48 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P < 0.05$, compared to the value of PGE_1 alone.

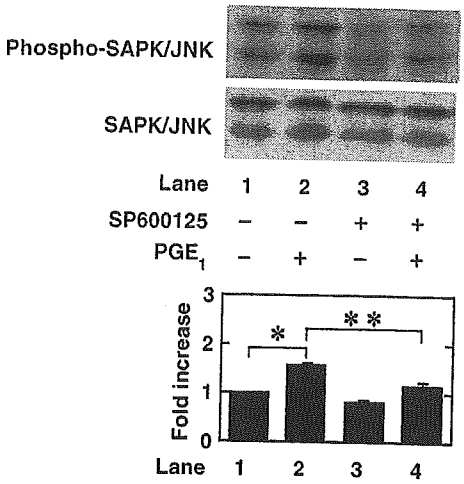

Fig. 4. Effect of SP600125 on the PGE_1-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM PGE_1 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of PGE_1-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. 'Fold increase' indicates the relative expression levels of phosphorylated SAPK/JNK after normalization with that of each total SAPK/JNK. $^*P < 0.05$, compared with the value of vehicle. $^{**}P < 0.05$, compared with the value of PGE_1 alone. Similar results were obtained with two additional and different cell preparations.

In order to further clarify that SP600125 is actually effective at inhibiting SAPK/JNK, we firstly examine the effect of PGE_1 on c-Jun phosphorylation. PGE_1 time-dependently induced the phosphorylation of c-Jun and the maximum effect of PGE_1 was observed at 60 min after the stimulation (data not shown). We next examined the effect of SP600125 on the PGE_1-induced c-Jun phosphorylation, and found that the phosphorylation of c-Jun stimulated by PGE_1 was suppressed by SP600125 (Fig. 5). SP600125 elicited about 45% reduction of the PGE_1-effect on the c-Jun phosphorylation.

3.4. Effect of forskolin on the phosphorylation of SAPK/JNK and effect of SP600125 on 8bromo-cAMP-induced VEGF synthesis in MC3T3-E1 cells

We have previously shown that PGE_1 activates adenylyl cyclase, resulting in the formation of cAMP in osteoblast-like MC3T3-E1 cells and that PGE_1 activates both p44/p42 MAP kinase and p38 MAP kinase via cAMP-dependent protein kinase (protein kinase A) in these cells (Ito et al., 1996; Tokuda et al., 2001). We next examined the effect of forskolin, a direct activator of adenylyl cyclase (Seamon and Daly, 1981), on the phosphorylation of SAPK/JNK in these cells. Forskolin-induced time-dependent phosphorylation of SAPK/JNK was seen up to 20 min, after which it decreased (Fig. 6).

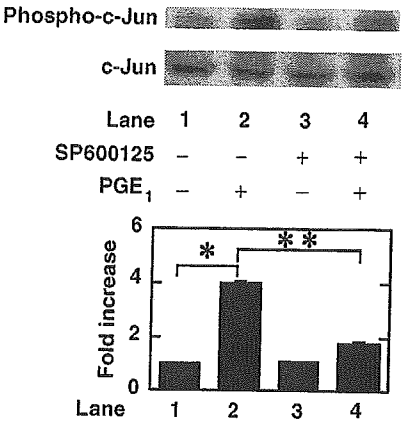

Fig. 5. Effect of SP600125 on the PGE_1-induced phosphorylation of c-Jun in MC3T3-E1 cells. The cultured cells were pretreated with 3 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM PGE_1 or vehicle for 60 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific c-Jun or c-Jun. The histogram shows quantitative representations of the levels of PGE_1-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. 'Fold increase' indicates the relative expression levels of phosphorylated c-Jun after normalization with that of each total c-Jun. $^*P < 0.05$, compared with the value of vehicle. $^{**}P < 0.05$, compared with the value of PGE_1 alone. Similar results were obtained with two additional and different cell preparations.

In addition, we examined the effect of SP600125 on the VEGF synthesis induced by 8bromo-cAMP, a plasma membrane-permeable cAMP analogue. We have demonstrated that 8bromo-cAMP alone stimulates VEGF synthesis in MC3T3-E1 cells (Tokuda et al., 2001). SP600125 significantly reduced the 8bromo-cAMP-induced VEGF synthesis (Table 1).

3.5. Effect of SP600125 on the phosphorylation of p38 MAP kinase induced by PGE_1 in MC3T3-E1 cells

SP600125 did not suppress the PGE_1-induced phosphorylation of p38 MAP kinase (Fig. 7). According to the densitometric analysis, SP600125 slightly but significantly enhanced the phosphorylation of p38 MAP kinase.

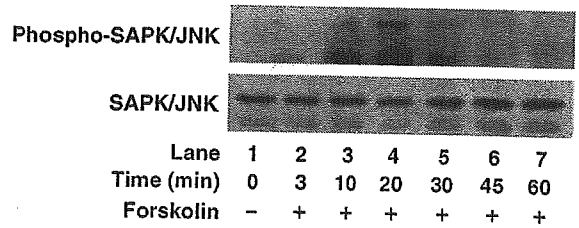

Fig. 6. Effect of forskolin on the phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were stimulated by 50 μM forskolin for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The upper bands and the lower ones indicated p54 and p46 SAPK/JNK, respectively. Lane 1, control cells.

Table 1
Effect of SP600125 on the 8bromo-cAMP-stimulated VEGF synthesis in MC3T3-E1 cells

SP600125	8bromo-cAMP	VEGF (pg/ml)
−	−	14 ± 2
−	+	258 ± 23
+	−	13 ± 2
+	+	173 ± 14*

The cultured cells were pretreated with 30 μM SP600125 or vehicle for 60 min, and then stimulated by 0.3 mM 8bromo-cAMP or vehicle for 24 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P < 0.05$, compared to the value of 8bromo-cAMP alone.

3.6. Effect of SB203580 on the PGE_1-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells

We previously reported that SB203580, a specific inhibitor of p38 MAP kinase (Cuenda et al., 1995), significantly reduced both VEGF synthesis and the phosphorylation of p38 MAP kinase induced by PGE_1 in MC3T3-E1 cells (Tokuda et al., 2001). Therefore, we examined the effect of SB203580 on the PGE_1 induced SAPK/JNK phosphorylation in these cells. However, SB203580 did not reduce the PGE_1 induced phosphorylation of SAPK/JNK (Fig. 8).

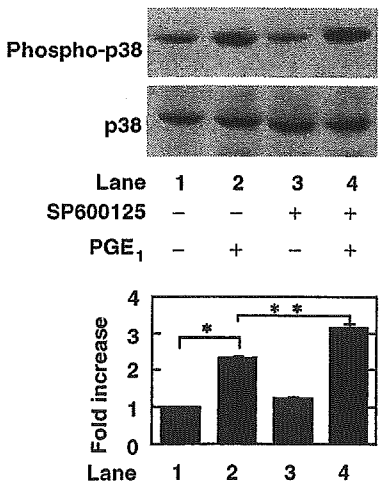

Fig. 7. Effect of SP600125 on the PGE_1-induced phosphorylation of p38 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 10 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM PGE_1 or vehicle for 10 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p38 MAP kinase or p38 MAP kinase. The histogram shows quantitative representations of the levels of PGE_1-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. 'Fold increase' indicates the relative expression levels of phosphorylated p38 MAP kinase after normalization with that of each total p38 MAP kinase. *$P < 0.05$, compared with the value of vehicle. **$P < 0.05$, compared with the value of PGE_1 alone. Similar results were obtained with two additional and different cell preparations.

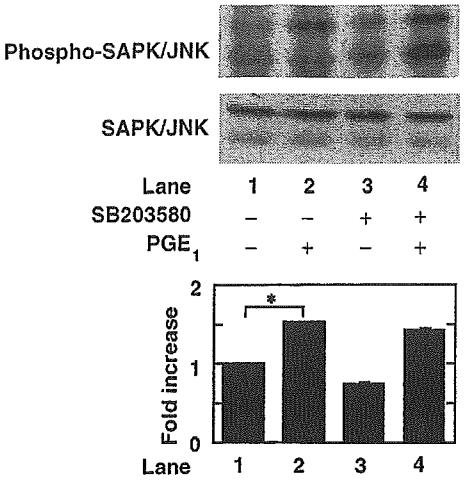

Fig. 8. Effect of SB203580 on the PGE_1-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SB203580 or vehicle for 60 min, and then stimulated by 10 μM PGE_1 or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of PGE_1-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± S.E.M. of triplicate determinations. 'Fold increase' indicates the relative expression levels of phosphorylated SAPK/JNK after normalization with that of each total SAPK/JNK. *$P < 0.05$, compared with the value of vehicle. Similar results were obtained with two additional and different cell preparations.

3.7. Effect of a combination of SP600125 and SB203580 on the PGE_1-stimulated VEGF synthesis in MC3T3-E1 cells

We further examined the effect of a combination of SP600125 and SB203580 on the PGE_1-stimulated VEGF synthesis in MC3T3-E1 cells. A combination of SP600125 and SB203580 significantly reduced the PGE_1-stimulated VEGF synthesis in an additive manner (Table 2).

Table 2
Effect of a combination of SP600125 and SB203580 on the PGE_1-stimulated VEGF synthesis in MC3T3-E1 cells

SP600125	SB203580	PGE_1	VEGF (pg/ml)
−	−	−	16 ± 2
−	−	+	842 ± 75
−	+	−	13 ± 3
−	+	+	308 ± 27*
+	−	−	14 ± 2
+	−	+	435 ± 38*
+	+	−	13 ± 2
+	+	+	55 ± 5**

The cultured cells were pretreated with 10 μM SP600125, 30 μM SB203580 or vehicle for 60 min, and then stimulated by 10 mM PGE_1 or vehicle for 48 h. Each value represents the mean ± S.E.M. of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

* $P < 0.05$, compared to the value of PGE_1 alone.

** $P < 0.05$, compared to the value of PGE_1 with SB203580 or SP600125.

4. Discussion

In the present study, we demonstrated that PGE_1 induces the phosphorylation of SAPK/JNK in osteoblast-like MC3T3-E1 cells. It is currently understood that the MAP kinase superfamily mediates intracellular signaling of extracellular agonists and plays a crucial role in cellular functions including proliferation, differentiation and cell death in a variety of cells (Widmann et al., 1999). Three major MAP kinase, p44/p42 MAP kinase, p38 MAP kinase, and SAPK/JNK are known as central elements used by mammalian cells to transducer the diverse messages (Widmann et al., 1999). We have previously shown that PGE_1 activates both p44/p42 MAP kinase and p38 MAP kinase in MC3T3-E1 cells (Tokuda et al., 2001). It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase (Raingeaud et al., 1995; Widmann et al., 1999). Therefore, these results strongly suggest that PGE_1 activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in osteoblast-like MC3T3-E1 cells. This is probably the first report showing the PGE_1-induced SAPK/JNK activation in osteoblasts as far as we know.

Herein, we showed that SP600125, a specific inhibitor of SAPK/JNK (Bennett et al., 2001), significantly suppressed the PGE_1-induced VEGF synthesis in osteoblast-like MC3T3-E1 cells. In addition, we found that SP600125 truly attenuated the phosphorylation of SAPK/JNK induced by PGE_1 in these cells. We previously reported that activation of p38 MAP kinase plays as a positive regulator in the PGE_1-induced VEGF synthesis in MC3T3-E1 cells (Tokuda et al., 2001). However, SP600125 did not suppress but rather enhanced the PGE_1-induced phosphorylation of p38 MAP kinase. On the other hand, we here showed that SP600125 reduced the c-Jun phosphorylation elicited by PGE_1 in these cells. Thus, it is probable that the suppression by SP600125 of PGE_1-stimulated VEGF synthesis is truly due to the inhibition of SAPK/JNK activation in MC3T3-E1 cells. These results strongly suggest that the activation of SAPK/JNK is involved in PGE_1-stimulated VEGF synthesis in osteoblast-like MC3T3-E1 cells. In addition, we showed that SB203580, a specific inhibitor of p38 MAP kinase (Cuenda et al., 1995), did not reduce the levels of the phosphorylation of PGE_1-induced SAPK/JNK. Furthermore, the PGE_1-stimulated VEGF synthesis was suppressed additively by a combination of SP600125 and SB203580. Therefore, our findings suggest that SAPK/JNK participates at least in part in the PGE_1-stimulated VEGF synthesis independently of p38 MAP kinase. Taking these findings into account, it is most likely that the VEGF synthesis stimulated by PGE_1 is mediated through the activation of both p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells.

It has been reported that PGE_1 increases mRNA for VEGF and the synthesis through the cAMP production in primary cultured rat calvaria cells and RCT-3 osteoblast-like cells (Harada et al., 1994). We previously reported that PGE_1 induces VEGF synthesis via activation of protein kinase A in osteoblast-like MC3T3-E1 cells (Tokuda et al., 2001). Herein, we demonstrated that forskolin, a direct activator of adenylyl cyclase (Seamon and Daly, 1981), induced the phosphorylation of SAPK/JNK in these cells, and that SP600125 truly reduced the 8bromo-cAMP-stimulated VEGF synthesis. These results suggest that SAPK/JNK acts at a point downstream from protein kinase A, and plays a pivotal role in PGE_1-induced VEGF synthesis. Based on our findings as a whole, it is most likely that PGE_1 induces VEGF synthesis through the protein kinase A-dependent activation of both p38 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells.

The expansion of capillary network providing microvasculature is an essential process of bone remodeling (Erlebacher et al., 1995). Since VEGF is a specific mitogen of vascular endothelial cells (Ferrara and Davis-Smyth, 1997), it is probable that VEGF synthesized by osteoblasts acts as an important intercellular mediator between osteoblasts and vascular endothelial cells. Moreover, it has been reported that VEGF is involved in trabecular bone formation and expansion of the hypertrophic chondrocyte zone in epiphyseal growth plate of mouse (Gerber et al., 1999), supporting the importance of VEGF in bone metabolism. PGs are known to be autocrine/paracrine modulators of osteoblasts (Nijweide et al., 1986, Smith, 1986). It is well established that PGs of the E series are the most potent activators of bone resorption in organ cultures (Pilbeam et al., 2002). The clinical use of aspirin or non-steroidal anti-inflammatory drugs, which inhibits the PGs synthesis, has been found to be associated with significant increase in bone mineral density (Bauer et al., 1996). In addition, it is recognized that both resorption and formation of bone are impaired in cyclooxygenase-2 null mice (Pilbeam et al., 2002). These findings indicate the crucial roles of PGs in the bone remodeling process. Although PGE_1 is not a physiological but a synthetic prostaglandin, the effect is known to be mediated through EP receptors (Narumiya et al., 1999). Among them, EP_2 and EP_4 are known to be coupled to increase cAMP levels (Narumiya et al., 1999), and both of them reportedly exist in MC3T3-E1 cells (Suda et al., 1996). Using the pharmacological tools, we have previously shown that not EP_2 but EP_4 possibly mediates the PGE_1-induced VEGF synthesis in these cells (Tokuda et al., 2001). From the knock-out mice studies (Li et al., 2000; Miyaura et al., 2000), it is recognized that EP_2 and EP_4 in osteoblasts are involved in the osteoclastogenesis, resulting in bone resorption. In addition to the well-established role to migrate vascular endothelial cells, it has recently been reported that VEGF induces osteoclast chemotaxix (Henriksen et al., 2003). Taking these findings into account as a whole, it is probable that VEGF synthesis by osteoblasts through the stimulation of EP_4 and/or EP_2 plays an important role in the bone remodeling process even under the physiological conditions. Further investigations would be required to clarify the details.

In conclusion, our present results strongly suggest that PGE_1 activates SAPK/JNK in addition to p44/p42 MAP kinase and p38 MAP kinase in osteoblasts, and that SAPK/JNK plays as a positive regulator at least in part in PGE_1-induced VEGF synthesis.

Acknowledgements

This investigation was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan.

References

Bauer, D.C., Orwoll, E.S., Fox, K.M., Vogt, T.M., Lane, N.E., Hochberg, M.C., Stone, K., Nevitt, M.C., 1996. Aspirin and NSAID use in older women: effect on bone mineral density and fracture risk. Study of Osteoporotic Fractures Research Group. J. Bone Miner. Res. 11, 29–35.

Bennett, B.L., Sasaki, D.T., Murray, B.W., O'Leary, E.C., Sakata, S.T., Xu, W., Leisten, J.C., Motiwala, A., Pierce, S., Satoh, Y., Bhagwat, S.S., Manning, A.M., Anderson, D.W., 2001. SP600125, an anthrapyrazolone inhibitor of Jun N-terminal Kinase. Proc. Natl. Acad. Sci. U.S.A. 98, 13681–13686.

Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R., Lee, J.C., 1995. SB203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. FEBS Lett. 364, 229–233.

Erlebacher, A., Filvaroff, E.H., Girelman, S.E., Derynck, R., 1995. Toward a molecular understanding of skeletal development. Cell 80, 371–378.

Ferrara, N., Davis-Smyth, T., 1997. The biology of vascular endothelial growth factor. Endocr. Rev. 18, 4–25.

Gerber, H.-P., Vu, T.H., Ryan, A.M., Kowalski, J., Werb, Z., Ferrara, N., 1999. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat. Med. 5, 623–628.

Goad, D.L., Rubin, J., Wang, H., Tashijian Jr., A.H., Patterson, C., 1996. Enhanced expression of vascular endothelial growth factor in human SaOS-2 osteoblast-like cells and murine osteoblasts induced by insulin-like growth factor I. Endocrinology 137, 2262–2268.

Harada, S., Nagy, J.A., Sullivan, K.A., Thomas, K.A., Endo, N., Rodan, G.A., 1994. Induction of vascular endothelial growth factor expression by prostaglandin E_2 and E_1 in osteoblasts. J. Clin. Invest. 93, 2490–2496.

Henriksen, K., Karsdal, M., Delaiss, J.-M., Engsig, M.T., 2003. RANKL and vascular endothelial growth factor (VEGF) induce osteoclast chemotaxis through an ERK1/2-dependent mechanism. J. Biol. Chem. 278, 48745–48753.

Ito, Y., Suzuki, A., Watanabe-Tomita, Y., Oiso, Y., Kozawa, O., 1996. Okadaic acid enhances prostaglandin E_1 induced alkaline phosphatase activity in osteoblast-like cells: regulation at a point downstream from protein kinase A. Prostaglandins Leukot. Essent. Fatty Acids 55, 357–361.

Kato, K., Ito, H., Hasegawa, K., Inaguma, Y., Kozawa, O., Asano, T., 1996. Modulation of the stress-induced synthesis of hsp27 and αB-crystallin by cyclic AMP in C6 glioma cells. J. Neurochem. 66, 946–950.

Kozawa, O., Tokuda, H., Miwa, M., Kotoyori, J., Oiso, Y., 1992. Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells. Exp. Cell Res. 198, 130–134.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Li, X., Okada, Y., Pilbeam, C.C., Lorenzo, J.A., Kennedy, C.R.J., Breyer, R.M., Raisz, L.G., 2000. Knockout of the murine prostaglandin EP_2 receptor impairs osteoclastogenesis in vitro. Endocrinology 141, 2054–2061.

Miyaura, C., Inada, M., Suzawa, T., Sugimoto, Y., Ushikubi, F., Ichikawa, A., Narumiya, S., Suda, T., 2000. Impaired bone resorption to prostaglandin E receptor EP4-knockout mice. J. Biol. Chem. 275, 19819–19823.

Narumiya, S., Sugimoto, Y., Ushikubi, F., 1999. Prostanoid receptors: structures properties and functions. Physiol. Rev. 79, 1193–1226.

Nijweide, P.J., Burger, E.H., Feyen, J.H.M., 1986. Cells of bone: proliferation, differentiation, and humoral regulation. Physiol. Rev. 66, 855–886.

Pilbeam, C.C., Harrison, J.R., Raisz, L.G., 1996. Prostaglandins and bone metabolism. In: Bilezikian, J.P., Raisz, L.G., Rodan, G.A. (Eds.), Principles of Bone Biology. Academic Press Inc., San Diego, pp. 715–728.

Pilbeam, C.C., Harrison, J.R., Raisz, L.G., 2002. Prostaglandins and bone metabolism. In: Bilezikian, J.P., Raisz, L.G., Rodan, G.A. (Eds.), Principles of Bone Biology, second ed. Academic Press Inc., San Diego, pp. 979–994.

Raingeaud, J., Gupta, S., Rogers, J.S., Dickens, M., Han, J., Ulevitch, R.J., David, R.J., 1995. Pro-inflammatory cytokines and enviromental stress cause p38 MAP kinase activation by dual phosphorylation on tyrosine and threonine. J. Biol. Chem. 270, 7420–7426.

Robinson, R.J., Doty, S.B., Cooper, R.R., 1973. Electron microscopy of mammalian bone. In: Zipkin, I. (Ed.), Biological Mineralization. Academic Press Inc., New York, pp. 257–296.

Schalaeppi, J.M., Gutzwiller, S., Finlenzeller, G., Fournier, B., 1997. 1,25-dihydroxyvitamin D_3 induces the expression of vascular endothelial growth factor in osteoblastic cells. Endocr. Res. 23, 213–229.

Seamon, K.B., Daly, J.W., 1981. Forskolin: a unique diterpene activator of cyclic AMP-generating systems. J. Cyclic Nucleotide Res. 7, 201–224.

Smith, W.L., 1986. The eicosanoids and their biochemical mechanism of action. Biochem. J. 259, 315–324.

Suda, M., Tanaka, K., Natsui, K., Uui, T., Tanaka, I., Fukushima, M., Shigeno, C., Konishi, J., Narumiya, S., Ichikawa, A., Nakao, N., 1996. Prostaglandin E receptor subtypes in mouse osteoblastic cell line. Endocrinology 137, 1698–1705.

Sudo, H., Kodama, H., Amagai, Y., Yamamoto, S., Kasai, S., 1983. In vivo differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria. J. Cell Biol. 96, 191–198.

Tokuda, H., Kozawa, O., Miwa, M., Uematsu, T., 2001. p38 Mitogen-activated protein (MAP) kinase but not p44/p42 MAP kinase is involved in prostaglandin E1-induced vascular endothelial growth factor (VEGF) synthesis in osteoblasts. J. Endocrinol. 170, 629–638.

Wang, D.S., Yamazaki, K., Nohtomi, K., Shizume, K., Ohsumi, K., Shibuya, M., Demura, H., Sato, K., 1996. Increase of vascular endothelial growth factor mRNA expression by 1,25-dihydroxyvitamin D_3 in human osteoblast-like cells. J. Bone Miner. Res. 11, 472–479.

Widmann, C., Gibson, S., Jarpe, M.B., Johnson, G.L., 1999. Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human. Physiol. Rev. 79, 143–180.

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Involvement of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) in prostaglandin $F_{2\alpha}$-induced heat shock protein 27 in osteoblasts

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Received 23 June 2003; accepted 18 September 2003

Abstract

We have reported that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) activates p44/p42 mitogen-activated protein (MAP) kinase in osteoblast-like MC3T3-E1 cells, and that p44/p42 MAP kinase plays a role in the $PGF_{2\alpha}$-induced heat shock protein 27 (HSP27). In the present study, we investigated the involvement of stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK), a member of the MAP kinase superfamily, in $PGF_{2\alpha}$-induced HSP27 in MC3T3-E1 cells. $PGF_{2\alpha}$ time dependently induced the phosphorylation of SAPK/JNK. SP600125, a specific inhibitor of SAPK/JNK, markedly reduced the $PGF_{2\alpha}$-stimulated HSP27 accumulation. The inhibitory effect of SP600125 was dose dependent in the range between 0.1 and 30 μM. SP600125 reduced the $PGF_{2\alpha}$-increased level of HSP27 mRNA. SP600125 suppressed the phosphorylation of SAPK/JNK induced by $PGF_{2\alpha}$, but did not affect the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase. On the other hand, PD98059, a specific inhibitor of the upstream kinase of p44/p42 MAP kinase, which reduced the phosphorylation of p44/p42 MAP kinase stimulated by $PGF_{2\alpha}$, had little effect on the $PGF_{2\alpha}$-induced phosphorylation of SAPK/JNK. These results strongly suggest that SAPK/JNK plays a part in $PGF_{2\alpha}$-induced HSP27 in addition to p44/p42 MAP kinase in osteoblasts.

Keywords: $PGF_{2\alpha}$; HSP27; SAPK/JNK; Osteoblast

1. Introduction

Heat shock proteins (HSPs) are produced by cells when the cells are exposed to the biological stress such as heat stress and chemical stress [1]. HSPs are classified into high- and low-molecular-weight HSPs based on apparent molecular sizes. It is well known that the high-molecular-weight HSPs such as HSP90 and HSP70 act as molecular chaperones in protein folding, oligomerization and translocation [1]. Low-molecular-weight HSPs with molecular masses from 10 to 30 kDa, such as HSP27 and αB-crystallin, have high homology in amino acid sequences [1]. It is currently recognized that the low-molecular-weight HSPs may have chaperoning functions like the high-molecular-weight HSPs [1]. Bone metabolism is regulated by two functional cells, osteoblasts and osteoclasts, responsible for bone formation and bone resorption, respectively [2]. The formation of bone structures and bone remodeling results from the coupling process, bone resorption by activated osteoblasts with subsequent deposition of new matrix by osteoblasts. Accumulating evidence indicates that osteoblasts are responsible for bone resorptive factors such as parathyroid hormone and 1.25-$(OH)_2$ vitamin D_3 [2] through the upregulation of RANKL expression [3], suggesting that osteoblasts play crucial role in the regulation of bone remodeling. In osteoblasts, the expression of HSP27 is induced by heat, and the heat-induced HSP27 expression is reportedly facilitated by estrogen [4,5]. In addition, it has been shown that the downregulation of proliferation is accompanied by a transient increase of the expression of HSP27 mRNA [4,5]. We have reported that physiological agents for bone metabolism such as endothelin-1 and transforming

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doi:10.1016/j.plefa.2003.09.006

growth factor β stimulate the induction of HSP27 in osteoblast-like MC3T3-E1 cells [6,7]. However, the exact mechanism behind the HSP27 induction in osteoblasts and its roles have not yet been precisely clarified.

Prostaglandin (PG) $F_{2\alpha}$, known as a potent bone resorptive agent, stimulates the proliferation and inhibits the differentiation of osteoblasts [8]. We have previously reported that $PGF_{2\alpha}$ activates phosphoinositide-specific phospholipase C (PI-PLC) and phosphatidylcholine-specific phospholipase D (PC-PLD) in osteoblast-like MC3T3-E1 cells, resulting in the protein kinase C activation [9,10]. In addition, we have reported that $PGF_{2\alpha}$ stimulates the induction of HSP27 through the PKC-dependent activation of p44/p42 mitogen-activated protein (MAP) kinase in these cells [11]. In the present study, we investigated the involvement of SAPK/JNK in the $PGF_{2\alpha}$-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells. We here show that SAPK/JNK in addition to p44/p42 MAP kinase acts as a positive regulator in $PGF_{2\alpha}$-induced HSP27 in these cells.

2. Materials and methods

2.1. Materials

$PGF_{2\alpha}$ was purchased from Sigma (St. Louis, MO). SP600125 and PD98059 were obtained from Calbiochem–Novabiochem Co. (La Jolla, CA). Phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p44/p42 MAP kinase antibodies and p44/p42 MAP kinase antibodies were obtained from New England BioLabs (Beverly, MA). An ECL Western blotting detection system was obtained from Amersham, Japan (Tokyo, Japan). Other materials and chemicals were obtained from commercial sources. SP600125 and PD98059 were dissolved in dimethyl sulfoxide. The maximum concentration of dimethyl sulfoxide was 0.1%, which did not affect immunoassay for HSP27, Northern blot analysis or Western blot analysis.

2.2. Cell culture

Cloned osteoblast-like MC3T3-E1 cells which have been derived from newborn mouse calvaria [12] were maintained as previously described [13]. Briefly, the cells were cultured in α-minimum essential medium (α-MEM) containing 10% fetal calf serum (FCS) at 37°C in a humidified atmosphere of 5% CO_2/95% air. The cells were seeded into 35-mm diameter dishes (5×10^4 cells/dish) or 90-mm diameter dishes (5×10^5 cells/dish) in α-MEM containing 10% FCS. After 5 days, the medium was exchanged for α-MEM containing 0.3% FCS. The cells were used for experiments after 48 h. When indicated, the cells were pretreated with SP600125 or PD98059 for 60 min.

2.3. Immunoassay of HSP27

The concentration of HSP27 in soluble extracts of the cells was determined by means of a sandwich-type enzyme immunoassay, as described previously [14]. The cultured cells were stimulated by $PGF_{2\alpha}$ for the indicated periods in 1 ml of α-MEM containing 0.3% FCS. The cells were washed twice with 1 ml of phosphate-buffered saline and frozen at −80°C for a few days before analysis. The frozen cells on each dish were collected and suspended in 0.3 ml of phosphate-buffered saline, and then each suspension was sonicated and centrifuged at 125,000 *g* for 20 min at 4°C. The supernatant was used for the immunoassay that employs polystyrene balls (3.2 mm in diameter; Immuno Chemicals, Okayama, Japan) carrying immobilized $F(ab')_2$ fragments of antibody and the same Fab′ fragments labeled with β-D-galactosidase from *Escherichia coli*. A polystyrene ball carrying antibodies was incubated either with the purified standard for HSP27 or with an aliquot of the samples. The incubation was carried out at 30°C for 5 h in a final volume of 0.5 ml of 10 mM sodium phosphate buffer, pH 7.0, containing 0.3 M NaCl, 0.5% hydrolyzed gelatin, 0.1% bovine serum albumin (BSA), 1 mM $MgCl_2$, and 0.1% NaN_3. After being washed, each ball was incubated at 4°C overnight with 1.5 mU of galactosidase-labeled antibodies in a volume of 0.2 ml with 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 M NaCl, 1 mM $MgCl_2$, 0.1% BSA, and 0.1% NaN_3. The galactosidase activity bound to the ball was assayed using a fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside.

2.4. Isolation of RNA and Northern blotting analysis of HSP27

The cultured cells were stimulated by $PGF_{2\alpha}$ in serum-free α-MEM for the indicated periods. Total RNA was isolated using a QuickPrep Total RNA Extraction kit (Pharmacia Biotech, Tokyo, Japan). Next, 20 μg of total RNA were subjected to electrophoresis on a 0.9% agarose-2.2 M formaldehyde gel and were blotted onto a nitrocellulose membrane. For Northern blot analysis, membrane was allowed to hybridize with a cDNA probe that had been labeled with a multiprime DNA labeling system (Amersham, Buckinghamshire, UK), as described previously [15]. A *Bam*HI–*Hind*III fragment of cDNA for mouse HSP27 [14] was kindly provided by Dr. L.F. Cooper of the University of North Carolina.

2.5. Western blot analysis of SAPK/JNK and p44/p42 MAP kinase

The cultured cells were stimulated by $PGF_{2\alpha}$ in serum-free α-MEM for the indicated periods. The cells were washed twice with phosphate-buffered saline and then lysed, homogenized and sonicated in a lysis buffer containing 62.5 mM Tris/Cl, pH 6.8, 2% sodium dodecyl sulfate (SDS), 50 mM dithiothreitol and 10% glycerol. The cytosolic fraction was collected as a supernatant after centrifugation at 125,000 *g* for 10 min at 4°C. SDS-polyacrylamide gel electrophoresis (PAGE) was performed by the method of Laemmli [16] in 10% polyacrylamide gels. Western blotting was performed as described previously [15] by using phospho-specific SAPK/JNK antibodies, SAPK/JNK antibodies, phospho-specific p44/p42 MAP kinase antibodies or p44/p42 MAP kinase antibodies, with peroxidase-labeled antibodies raised in goat against rabbit IgG being used as second antibodies. Peroxidase activity on the nitrocellulose sheet was visualized on X-ray film by use of an ECL Western blotting detection system.

2.6. Determination

The absorbance of enzyme immunoassay samples was measured at 450 nm with EL 340 Bio Kinetic Reader (Bio-Tek Instruments, Inc., Winooski, VT). The densitometric analysis was performed using Molecular Analyst/Macintosh (Bio-Rad Laboratories, Hercules, CA).

2.7. Statistical analysis

The data were analyzed by ANOVA followed by the Bonferroni method for multiple comparison between pairs, and a $P<0.05$ was considered significant. All data are presented as the mean ± SEM of triplicate determinations. Each experiment was repeated three times with similar results.

3. Results

3.1. Effect of $PGF_{2\alpha}$ on the phosphorylation of SAPK/JNK in MC3T3-E1 cells

To clarify whether $PGF_{2\alpha}$ activates SAPK/JNK in osteoblast-like MC3T3-E1 cells, we examined the effect of $PGF_{2\alpha}$ on the SAPK/JNK phosphorylation. $PGF_{2\alpha}$ markedly induced the phosphorylation of SAPK/JNK in a time-dependent manner up to 30 min (Fig. 1). The maximum effect of $PGF_{2\alpha}$ on the SAPK/JNK phosphorylation was observed at 20 min after the stimulation.

3.2. Effect of SP600125 on the $PGF_{2\alpha}$-stimulated HSP27 accumulation in MC3T3-E1 cells

To investigate whether SAPK/JNK is involved in the $PGF_{2\alpha}$-stimulated HSP27 induction in MC3T3-E1 cells, we examined the effect of SP600125, a highly specific inhibitor of SAPK/JNK [17], on the accumulation of HSP27. SP600125, which alone did not affect the HSP27 accumulation, significantly suppressed the $PGF_{2\alpha}$-stimulated HSP27 accumulation (Fig. 2). The inhibitory effect of SP600125 on the $PGF_{2\alpha}$-stimulated HSP27 accumulation was dose-dependent in the range between 0.1 and 30 μM (Fig. 3). The maximum effect of SP600125 on the HSP27 accumulation was observed at

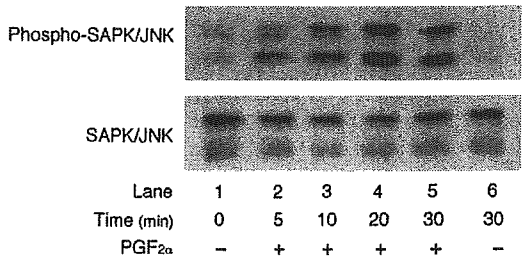

Fig. 1. Effect of $PGF_{2\alpha}$ on the phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for the indicated periods. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. Lane 1, unstimulated cells.

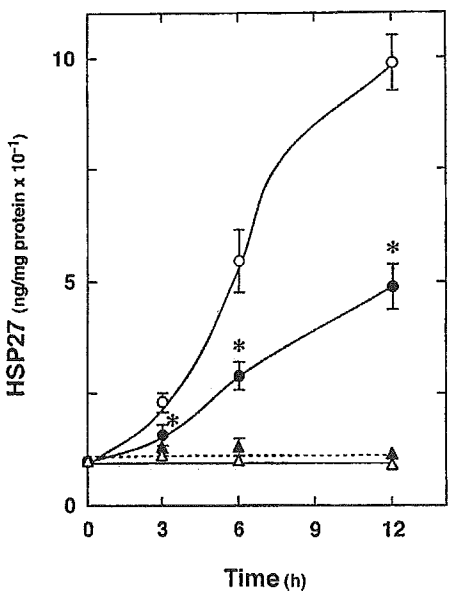

Fig. 2. Effect of SP600125 on the $PGF_{2\alpha}$-stimulated accumulation of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SP600125 (closed symbols) or vehicle (open symbols) for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ (circles) or vehicle (triangles) for the indicated periods. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P<0.05$, compared to the value of $PGF_{2\alpha}$ alone.

30 μM, which caused about 60% reduction in the $PGF_{2\alpha}$-effect.

3.3. *Effect of SP600125 on* $PGF_{2\alpha}$*-increased level of HSP27 mRNA in MC3T3-E1 cells*

We previously showed that the maximum effect of $PGF_{2\alpha}$ on the level of HSP27 mRNA is observed at 2 h after the stimulation [11]. We next examined the effect of SP600125 on the $PGF_{2\alpha}$-increased level of mRNA for HSP27 in MC3T3-E1 cells. SP600125 markedly reduced the $PGF_{2\alpha}$-increased level of HSP27 mRNA (Fig. 4).

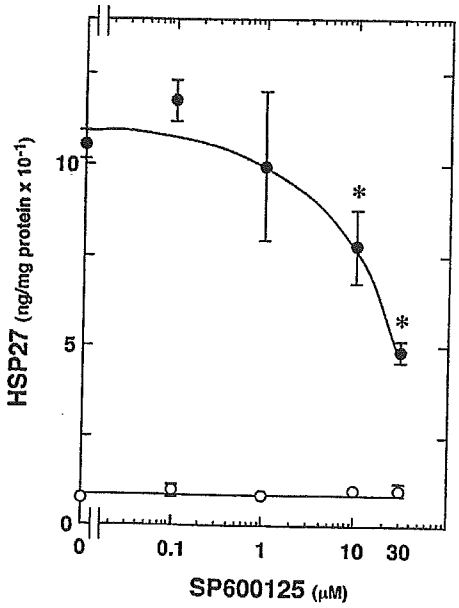

Fig. 3. Dose-dependent effect of SP600125 on the $PGF_{2\alpha}$-stimulated accumulation of HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with various doses of SP600125 for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ (closed symbols) or vehicle (open symbols) for 12 h. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P<0.05$, compared to the value of $PGF_{2\alpha}$ alone.

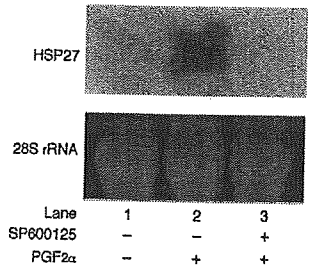

Fig. 4. Effect of SP600125 on the $PGF_{2\alpha}$-increased levels of the mRNA for HSP27 in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 2 h. The cells were harvested and total RNA was isolated. Twenty micrograms of RNA from each sample was subjected to electrophoresis and blotted onto a nitrocellulose membrane. The membrane was then allowed to hybridize with cDNA probe for HSP27. Bands of 28S rRNA are shown for reference.

3.4. *Effects of SP600125 on the phosphorylation of SAPK/JNK or p44/p42 MAP kinase induced by* $PGF_{2\alpha}$ *in MC3T3-E1 cells*

We found that SP600125 truly inhibited the phosphorylation of SAPK/JNK induced by $PGF_{2\alpha}$ (Fig. 5). According to the densitometric analysis, SP600125 almost completely reduced the $PGF_{2\alpha}$-effect on the SAPK/JNK phosphorylation. However, SP600125 had little effect on the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase (Fig. 6).

3.5. *Effects of PD98059 on the* $PGF_{2\alpha}$*-induced phosphorylation of p44/p42 MAP kinase or SAPK/JNK in MC3T3-E1 cells*

PD98059, an inhibitor of the upstream kinase of p44/p42 MAP kinase [18], significantly reduced the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase (Fig 7). On the other hand, PD98059 had little effect

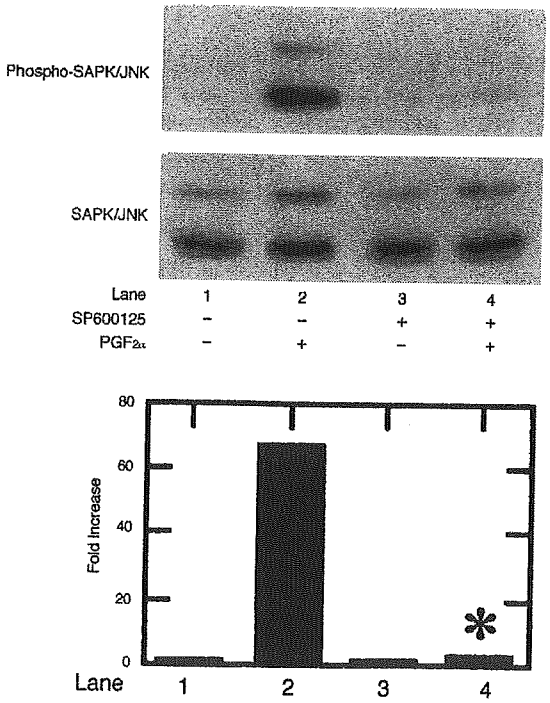

Fig. 5. Effect of SP600125 on the $PGF_{2\alpha}$-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^*P<0.05$, compared with the value of $PGF_{2\alpha}$ alone.

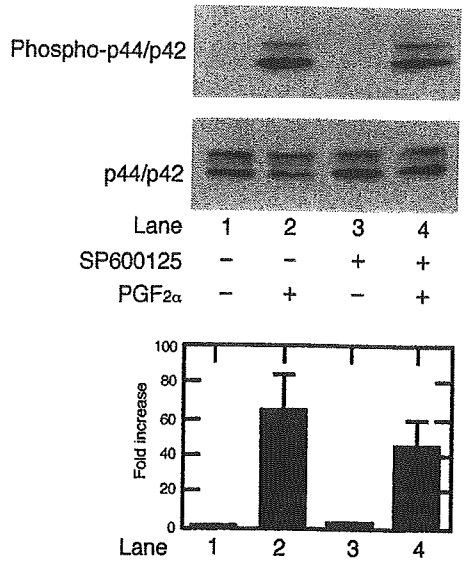

Fig. 6. Effect of SP600125 on the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM SP600125 or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

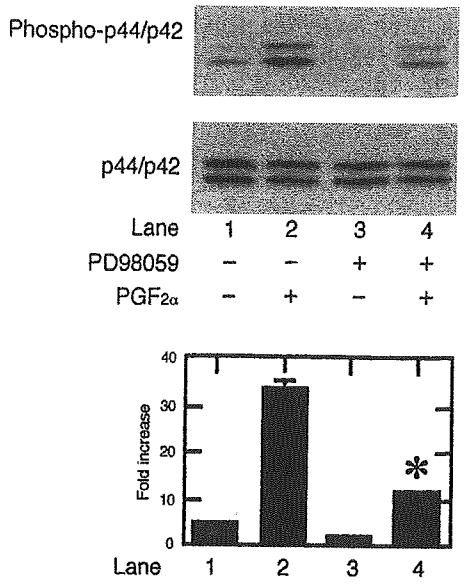

Fig. 7. Effect of PD98059 on the $PGF_{2\alpha}$-induced phosphorylation of p44/p42 MAP kinase in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD98059 or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific p44/p42 MAP kinase or p44/p42 MAP kinase. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations. $^{*}P<0.05$, compared with the value of $PGF_{2\alpha}$ alone.

on the $PGF_{2\alpha}$-induced phosphorylation of SAPK/JNK (Fig. 8).

4. Discussion

It is well known that the MAP kinase superfamily mediates intracellular signaling of various agonists and plays pivotal role in cellular functions including proliferation, differentiation and cell death in a variety of cells [19]. Three major MAP kinases such as p44/p42 MAP kinase, p38 MAP kinase and SAPK/JNK are recognized to transduce signals in mammalian cells [19]. We have previously reported that $PGF_{2\alpha}$-stimulates HSP27 induction through the activation of p44/p42 MAP kinase in osteoblast-like MC3T3-E1 cells [11]. Therefore, we conducted the present study to investigate whether SAPK/JNK plays a role in $PGF_{2\alpha}$-stimulated HSP27 induction in these cells.

Herein, we demonstrated that $PGF_{2\alpha}$ elicited the phosphorylation of SAPK/JNK in osteoblasts-like MC3T3-E1 cells. It is well recognized that MAP kinases are activated by phosphorylation of threonine and tyrosine residues by dual specificity MAP kinase kinase [19, 20]. Thus, it is most likely that $PGF_{2\alpha}$ activates SAPK/JNK in MC3T3-E1 cells. This is probably the

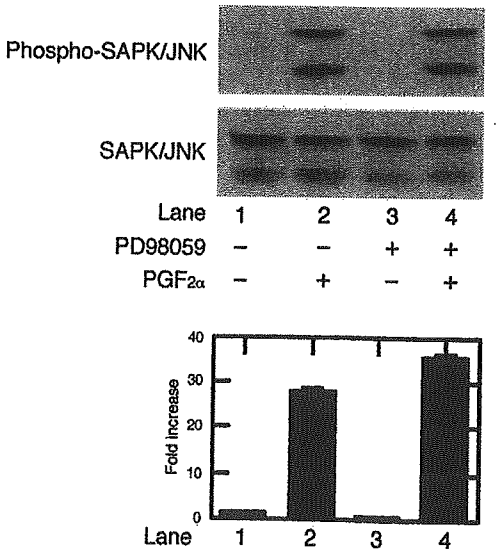

Fig. 8. Effect of PD98059 on the $PGF_{2\alpha}$-induced phosphorylation of SAPK/JNK in MC3T3-E1 cells. The cultured cells were pretreated with 30 μM PD98059 or vehicle for 60 min, and then stimulated by 10 μM $PGF_{2\alpha}$ or vehicle for 20 min. The extracts of cells were subjected to SDS-PAGE with subsequent Western blotting analysis with antibodies against phospho-specific SAPK/JNK or SAPK/JNK. The histogram shows quantitative representations of the levels of $PGF_{2\alpha}$-induced phosphorylation obtained from laser densitometric analysis of three independent experiments. Each value represents the mean ± SEM of triplicate determinations. Similar results were obtained with two additional and different cell preparations.

first report showing the activation by $PGF_{2\alpha}$ of SAPK/JNK in osteoblasts-like cells as far as we know. We also showed that SP600125 [17] significantly reduced the $PGF_{2\alpha}$-stimulated accumulation of HSP27 in MC3T3-E1 cells. Therefore, our findings suggest that SAPK/JNK is involved in the HSP27 induction by $PGF_{2\alpha}$ in these cells. In addition, we demonstrated that the phosphorylation induced by $PGF_{2\alpha}$ was truly suppressed by SP600125 in these cells. Furthermore, we found that SP600125 hardly affected the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$ in these cells. Thus, it is probable that SP600125 suppressed the accumulation of HSP27 induced by $PGF_{2\alpha}$ via the attenuation of not p44/p42 MAP kinase but SAPK/JNK in MC3T3-E1 cells. Additionally, the $PGF_{2\alpha}$-increased level of HSP27 mRNA was suppressed by SP600125. Taking our findings into account, it is most likely that SAPK/JNK plays a part in the $PGF_{2\alpha}$-stimulated HSP27 induction in osteoblast-like MC3T3-E1 cells.

We previously reported that PD98059 [18] attenuates the $PGF_{2\alpha}$-induced HSP27 accumulation in these cells. However, we found that PD98059, which significantly reduced the phosphorylation of p44/p42 MAP kinase induced by $PGF_{2\alpha}$, did not affect the phosphorylation of SAPK/JNK induced by $PGF_{2\alpha}$. Our results suggest that the attenuation by PD98059 of the $PGF_{2\alpha}$-induced HSP27 accumulation results from the suppression of not SAPK/JNK but p44/p42 MAP kinase activation in osteoblasts-like MC3T3-E1 cells. Based on our results as a whole, it is most likely that the HSP27 induction stimulated by $PGF_{2\alpha}$ is mediated through the activation of both p44/p42 MAP kinase and SAPK/JNK in osteoblast-like MC3T3-E1 cells. It is well known that the low-molecular-weight HSPs such as HSP27, as well as the high-molecular-weight HSPs such as HSP90 and HSP70, act as molecular chaperones in protein folding, oligomerization and translocation [1]. It is possible that $PGF_{2\alpha}$-induced accumulation of HSP27 plays a role in the changes of cellular function such as secretion, proliferation or differentiation in osteoblasts. To coordinate them, SAPK/JNK and p44/p42 MAP kinase seems to regulate cooperatively the induction of HSP27. In addition, it has been reported that the upregulation of HSP27 plays a role in cell survival pathways, resulting in increase of the resistance to apoptosis [21]. Taking these findings into account, it is most likely that $PGF_{2\alpha}$-increased HSP27 through the activation of SAPK/JNK in addition to p44/p42 MAP kinase finely coordinates the $PGF_{2\alpha}$-provocated cellular events to minimize the damage in osteoblasts. Further investigations would be required to clarify the exact roles of HSP27 in osteoblasts.

In conclusion, our present results strongly suggest that $PGF_{2\alpha}$-activated SAPK/JNK in addition to p44/p42 MAP kinase plays a part in $PGF_{2\alpha}$-induced HSP27 in osteoblasts.

Acknowledgements

This investigation was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. We are grateful to Y. Okamoto for her skillful secretarial assistance.

References

[1] I.J. Benjamin, D.R. McMillan, Stress (heat shock) proteins molecular chaperones in cardiovascular biology and diseases, Circ. Res. 83 (1998) 117–132.
[2] P.J. Nijweide, E.H. Burger, J.H. Feye, Cells of bone: proliferation, differentiation, and hormonal regulation, Physiol. Rev. 66 (1986) 855–886.
[3] H. Yasuda, N. Shima, N. Nakagawa, K. Yamaguchi, M. Kinosaki, S. Mochizuki, A. Tomoyasu, K. Yano, M. Goto, A. Murakami, N. Takahashi, T. Suda, Osteoclast differentiation factor is a ligand for osteoprotegerin/osteoclastgenesis-inhibitory factor and is identical to TRANCE/RANKL, Proc. Natl. Acad. Sci. USA 95 (1998) 3597–3602.
[4] A.R. Shakoori, A.M. Oberdorf, T.A. Owen, L.A. Weber, E. Hickey, J.L. Stein, J.B. Lian, G.S. Stein, Expression of heat shock genes during differentiation of mammalian osteoblasts and promyelocytic leukemia cells, J. Cell. Biochem. 48 (1992) 277–287.
[5] L.F. Cooper, K. Uoshima, Differential estrogenic regulation of small M(r) heat shock protein expression in osteoblasts, J. Biol. Chem. 269 (1994) 7869–7873.
[6] H. Kawamura, T. Otsuka, H. Matsuno, M. Niwa, N. Matsui, K. Kato, T. Uematsu, O. Kozawa, Endothelin-1 stimulates heat shock protein 27 induction in osteoblasts: involvement of p38 MAP kinase, Am. J. Physiol. 277 (1999) E1046–E1054.
[7] D. Hatakeyama, O. Kozawa, M. Niwa, H. Matsuno, H. Ito, K. Kato, N. Tatematsu, T. Shibata, T. Uematsu, Upregulation by retinoic acid of transforming growth factor-β-stimulated heat shock protein 27 induction in osteoblasts: involvement of mitogen-activated protein kinases, Biochim. Biophys. Acta. 1589 (2002) 15–30.
[8] C.C. Pilbeam, J.R. Harrison, L.G. Raisz, Prostaglandins and bone metabolism, in: J.P. Bilezikian, L.G. Raisz, G.A. Rodan (Eds.), Principles of Bone Biology, Academic Press, San Diego, 1996, pp. 715–728.
[9] M. Miwa, H. Tokuda, K. Tsushita, J. Kotoyori, Y. Takahashi, N. Ozaki, O. Kozawa, Y. Oiso, Involvement of pertussis toxin-sensitive GTP-binding protein in prostaglandin $F_{2\alpha}$-induced phosphoinositide hydrolysis in osteoblast-like cells, Biochem. Biophys. Res. Commun. 171 (1990) 1229–1235.
[10] O. Kozawa, A. Suzuki, J. Kotoyori, H. Tokuda, Y. Watanabe, Y. Ito, Y. Oiso, Prostaglandin $F_{2\alpha}$activates phospholipase D independently from activation of protein kinase C in osteoblast-like cells, J. Cell. Biochem. 55 (1994) 373–379.
[11] O. Kozawa, H. Tokuda, M. Miwa, H. Ito, H. Matsuno, M. Niwa, K. Kato, T. Uematsu, Involvement of p44/p42 mitogen-activated protein kinase in prostaglandin $F_{2\alpha}$-stimulated induction of heat shock protein 27 in osteoblasts, J. Cell. Biochem. 75 (1999) 610–619.
[12] H. Sudo, H. Kodama, Y. Amagai, S. Yamamoto, S. Kasai, In vivo differentiation and calcification in a new clonal osteogenic cell line derived from newborn mouse calvaria, J. Cell Biol. 96 (1983) 191–198.

[13] O. Kozawa, H. Tokuda, M. Miwa, J. Kotoyori, Y. Oiso, Cross-talk regulation between cyclic AMP production and phosphoinositide hydrolysis induced by prostaglandin E_2 in osteoblast-like cells, Exp. Cell Res. 198 (1992) 130–134.

[14] Y. Inaguma, S. Goto, H. Shinohara, K. Ohshima, K. Kato, Physiological and pathological changes in levels of the two small stress proteins, HSP27 and αB-crystallin in rat hindlimb muscles, J. Biochem. 114 (1993) 378–384.

[15] K. Kato, H. Ito, K. Hasegawa, Y. Inaguma, O. Kozawa, T. Asano, Modulation of the stress-induced synthesis of HSP27 and αB-crystallin by cyclic AMP in C6 rat glioma cells, J. Neurochem. 66 (1996) 946–950.

[16] U.K. Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227 (1970) 680–685.

[17] B.L. Bennett, D.T. Sasaki, B.W. Murray, E.C. O'Leary, S.T. Sakata, W. Xu, J.C. Leisten, A. Motiwala, S. Pierce, Y. Satoh, S.S. Bhagwat, A.M. Manning, D.W. Anderson, SP600125, an anthrapyrazolone inhibitor of Jun N-terminal Kinase, Proc. Natl. Acad. Sci. USA 98 (2001) 13681–13686.

[18] D.R. Alessi, A. Cuenda, P. Cohen, D.T. Dudley, A.R. Saltiel, PD98059 is a specific inhibitor of the activation of mitogen-activated protein kinase kinase in vitro and in vivo, J. Biol. Chem. 270 (1995) 27489–27494.

[19] C. Widmann, S. Gibson, M.B. Jarpe, G.L. Johnson, Mitogen-activated protein kinase: conservation of a three-kinase module from yeast to human, Physiol. Rev. 79 (1999) 143–180.

[20] J. Raingeaud, S. Gupta, J.S. Rogers, M. Dickens, J. Han, R.J. Ulevitch, R.J. David, Pro-inflammatory cytokines and environmental stress cause p38 MAP kinase activation by dual phosphorylation on tyrosine and threonine, J. Biol. Chem. 270 (1995) 7420–7426.

[21] M.J.D. Wagstaff, Y. Collaco-Moraes, J. Smith, J.S. de Belleroche, R.S. Coffin, D.S. Latchman, Protection of neuronal cells from apoptosis by HSP27 derived with a herpes simplex virus based vector, J. Biol. Chem. 274 (1999) 5061–5069.

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Activation of p38 mitogen-activated protein kinase mediates thyroid hormone-stimulated osteocalcin synthesis in osteoblasts

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Received 29 January 2003; accepted 14 October 2003

Abstract

It is well known that thyroid hormone modulates osteoblast cell function. We have previously shown that triiodothyronine (T_3) activates p44/p42 mitogen-activated protein (MAP) kinase, which limits T_3-induced alkaline phosphatase activity in osteoblast-like MC3T3-E1 cells. In the present study, we investigated whether p44/p42 MAP kinase or p38 MAP kinase is involved in the thyroid hormone-stimulated osteocalcin synthesis in these cells. T_3 markedly induced the phosphorylation of p38 MAP kinase in addition to p44/p42 MAP kinase. PD98059 and U0126, inhibitors of the upstream kinase that activates p44/p42 MAP kinase, had little effect on the T_3-induced synthesis of osteocalcin. On the contrary, the T_3-induced osteocalcin synthesis was significantly reduced by SB203580 and PD169316, inhibitors of p38 MAP kinase. SB203580, PD169316 or PD98059 suppressed the T_3-phosphorylation of myelin basic protein. T_3-induced osteocalcin synthesis was significantly reduced by SB203580 or PD169316 also in primary cultured mouse osteoblasts. These results strongly suggest that p38 MAP kinase but not p44/p42 MAP kinase takes part in the thyroid hormone-stimulated osteocalcin synthesis in osteoblasts.

Keywords: Thyroid hormone; Osteocalcin; Mitogen-activated protein kinase; Osteoblasts

1. Introduction

It is generally known that thyroid hormone is a crucial regulator of skeletal function, resulting in modulating bone metabolism and hyperthyroidism is a major cause of secondary osteoporosis (Khosla and Melton, 1995). In hyperthyroidism, the serum levels of alkaline phosphatase and osteocalcin, markers of osteoblast phenotype, and the excretion of pyridinoline and hydroxypyridinoline cross-link, which reflects bone resorption, are elevated (Stern, 1996) It is recognized that both increased bone resorption and decreased bone formation contribute to the loss of bone mass by hyperthyroidism (Stern, 1996). Bone metabolism is regulated mainly by two functional cells, osteoblasts and osteoclasts (Nijweide et al., 1986). The former cells are responsible for bone formation and the latter for bone resorption. The receptor for triiodothyronine (T_3) has been shown to exist on osteoblasts (Stern, 1996). It has been shown that thyroid hormone stimulates alkaline phosphatase activity and secretion of osteocalcin and insulin-like growth factors (IGFs) in osteoblasts and that it modulates proliferation of osteoblasts (Rizzoli et al., 1986; Kasono et al., 1988; Stern, 1996). IGF-I production is reportedly essential for the mitogenic effect of thyroid hormone but not for the stimulatory effects of thyroid hormone on alkaline phosphatase activity and osteocalcin synthesis in osteoblasts (Huang et al., 2000). In a previous study (Tokuda et al., 1998), we have reported that T_3 modulates interleukin-6 synthesis at two points in osteoblast-like MC3T3-E1 cells as follows; one is exerted at the point between adenylyl cyclase and protein kinase A, and the other is at a point downstream from protein kinase C activation. However, the exact mechanism of thyroid hormone in osteoblasts has not been precisely clarified.

The receptor of thyroid hormone belongs to the steroid hormone receptor superfamily (Evans, 1988). It is well recognized that the effects of thyroid hormone, as well as

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doi:10.1016/j.mce.2003.10.049